CELL-CELL RECOGNITION AND ADHESION DURING EMBRYOGENESIS IN THE SEA URCHIN: DEMONSTRATION OF SPECIES-SPECIFIC ADHESION AMONG *ARBACIA PUNCTULATA, LYTECHINUS VARIEGATUS,* AND *STRONGYLOCENTROTUS PURPURATUS*

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ABSTRACT

The present study investigates species-specific recognition and adhesion between dissociated embryonic cells of hatched blastulae of *Arbacia punctulata, Lytechinus variegatus,* and *Strongylocentrotus purpuratus.* The assay used is a modification of one previously developed by McClay and Hausman (1975) and involves collection of labeled single probe cells to unlabeled collecting aggregates. The results indicate that probe cells fixed by glutaraldehyde or formaldehyde or disrupted by sonication do not adhere significantly to their homospecific aggregates. Moreover, fixation of aggregates by glutaraldehyde greatly diminishes binding of labeled probe cells. Thus adhesion, as measured by the present assay, requires both living probe cells and aggregates. In addition, adhesion of probe cells to their homospecific aggregates is found to be significantly greater than adhesion to heterospecific aggregates. The results demonstrate reciprocal species-specific adhesion between *Arbacia punctulata versus Lytechinus variegatus, Arbacia punctulata versus Strongylocentrotus purpuratus,* and *Lytechinus variegatus versus Strongylocentrotus purpuratus.* These results extend previous work with other species and suggest that species-specific recognition and adhesion is a universal property of dissociated cells of sea urchin embryos.

INTRODUCTION

The pioneering studies of Herbst (1900) first showed that sea urchin embryos can be dissociated into separate cells in calcium-free sea water, and that these cells will reaggregate into embryo-like structures when returned to normal sea water. Later studies by Giudice (1962), Giudice and Mutolo (1970), Spiegel and Spiegel (1975), and McClay and Hausman (1975) have shown the reaggregation process to be species-specific between *Paracentrotus lividus* and *Arbacia lixula, Lytechinus pictus* and *Arbacia punctulata,* and *Tripneustes eschulentes* and *Lytechinus variegatus,* respectively. It is particularly noteworthy that species-specific adhesion is observed not only with dissociated cells from embryos at the hatched blastula through the prism stages (Giudice, 1962; Giudice and Mutolo, 1970; McClay and Hausman, 1975) but also with dissociated cells from blastulae prior to hatching (Giudice and Mutolo, 1970) and from embryos at the 16-cell stage (Spiegel and Spiegel, 1975). Although Giudice and Mutolo (1970) and Spiegel and Spiegel (1975) have reported loose adherence between species followed by "sorting out" when reaggregation occurs in stationary culture, a high

degree of species-specificity of adhesion is observed by Giudice (1962), McClay and Hausman (1975), and McClay *et al.* (1977) for reaggregation of cells in suspension culture. The apparent nonspecific association initially observed in stationary culture may result from factors influencing motility, chemotaxis, or cell to subtratum adhesion, rather than species-specific recognition and adhesion. Giudice (1962), Giudice and Mutolo (1970), Okazaki (1975), Sano (1977), and Spiegel and Spiegel (1980) have shown that embryonic cells do not appear to de-differentiate upon dissociation but rather they appear to retain the same pattern of metabolic activity and embryonic differentiation characteristic of the stage at which they were dissociated. Since Giudice and Mutolo (1970) have shown by autoradiography that dissociated cells of labeled blastulae will reaggregate with dissociated cells of prisms, it would appear that species-specificity rather than stage (or tissue) specificity may predominate initially in this system. Moreover, since Giudice and Mutolo (1970), Spiegel and Spiegel (1975, 1980), McClay and Hausman (1975) and others have demonstrated that reaggregation of dissociated cells can eventually lead to the formation of almost normal embryos, it appears that there is a further sorting out according to tissue after the initial species-specific event. Indeed previous experiments by Spiegel and Spiegel (1978) with isolated, vitally-stained micromeres support this idea. Undoubtedly, reaggregation of dissociated embryonic cells of the sea urchin involves many complex and multiple molecular interactions. However, it would seem quite probable that a recognition event involving species-specific adhesion might indeed be one of the earliest in a series of cell-cell interactions which lead to further sorting out (tissue-specific adhesion) and differentiation. The present work describes species-specific recognition and adhesion among sea urchin species readily available in this country—*Arbacia punctulata, Lytechinus variegatus,* and *Strongylocentrotus purpuratus*—by the use of a quantitative reaggregation assay. A portion of this work has appeared previously in abstract form (Schneider, 1983).

MATERIALS AND METHODS

For the studies performed in Woods Hole, Massachusetts, *Arbacia punctulata* were supplied by the Marine Biological Laboratory and *Lytechinus variegatus* were purchased from the Center for the Study of Cells, Reproduction, and Development (C.S.C.R.D.), Florida State University, Tallahassee, Florida. Animals were maintained in laboratory sea tables supplied with constantly flowing sea water (20–22°C). For the studies performed in Omaha, *A. punctulata* and *L. variegatus* were purchased from the C.S.C.R.D., Florida State, and *Strongylocentrotus purpuratus* were obtained from the Pacific Biomarine Corporation, Venice, California. The animals were maintained in refrigerated aquaria at 16°, 13°, and 21°C, respectively, in artificial sea water (Instant Ocean, from Aquarium Systems, Inc.; Mentor, Ohio). Gentamicin was obtained from the Schering Corporation. Prosil-28 was purchased from PCR Research Chemicals, Inc.; Gainesville, Florida. Paraformaldehyde was obtained from J. T. Baker; glutaraldehyde and L-leucine were supplied by Sigma. Leucine [L-4,5-^{3}H, 56.5 Ci/mmol] was purchased from New England Nuclear.

Fertilization of eggs and culturing of embryos

Gametes were obtained from the animals by intracoelomic injection with 0.5 M KCl (*S. purpuratus* and *L. variegatus*) or by electrical shock (*A. punctulata*). Eggs at concentrations of 0.5–1 mg of protein per ml (~1% suspension, v/v) were fertilized with a two to three-fold excess of sperm (0.02–0.3 μl of dry sperm per ml of eggs)

and washed several times by gravity sedimentation. Embryos were cultured with gentle stirring by a paddle wheel at 0.5–1 mg of protein per ml in artificial sea water (ASW: MBL formula; Harvey, 1956) containing 5 mM Tris-Cl, pH 8.0 and 10 μg/ml gentamicin. For studies at Woods Hole, *A. punctulata* and *L. variegatus* were cultured at 20–22°C. For studies in Omaha, *A. punctulata, L. variegatus,* and *S. purpuratus* were cultured at 16.5–19.5°C, 19.5°C, and 16.5°C, respectively.

Dissociation of embryos at the hatched blastula stage

Embryos at the hatched blastula stage (0.5–0.75 ml packed cell volume) were recovered by centrifugation by hand and resuspended at 0°C in 10 ml of calcium and magnesium-free sea water (CMFSW: 0.45 M NaCl, 0.01 M KCl, 0.025 M Na$_2$SO$_4$, and 0.0025 M NaHCO$_3$) containing 5 mM Tris-Cl, pH 8.0, and 1 mM EDTA. Embryos of *A. punctulata* were washed 3–4 times in this medium; those of *L. variegatus* and *S. purpuratus* were washed twice. The embryos were resuspended in 2 ml of CMFSW containing Tris and EDTA and gently dissociated at 0°C by repeated passages through a 5$\frac{3}{4}$″ Pasteur pipette. When dissociation was complete, as assessed by examination in a phase contrast microscope, the single cells were recovered by centrifugation at 0–5°C for 3 min at 1200 rpm in a Sorvall RC-3 clinical centrifuge.

Preparation of collecting aggregates

Dissociated cells from hatched blastulae were washed in 3 ml of CMFSW at 0°C, centrifuged at 1200 rpm for 3 min, and resuspended in 6 ml of CMFSW to a concentration of 5–10 mg of protein per ml (about 6–13 × 10^7 cells/ml). These were diluted ten-fold into plastic Petri dishes (Bellco, 100 × 20 mm) containing 16 ml ASW plus gentamicin. Dissociated cells were allowed to reaggregate undisturbed in stationary culture. Cultures of *A. punctulata* and *L. variegatus* cells were incubated at 20–22°C in Woods Hole and at 16.5°–18°C and 18°–20°C, respectively, in Omaha. Cultures of *S. purpuratus* cells were incubated at 16.5°–18°C. Dissociated cells from hatched blastulae exhibited varying degrees of initial adhesion to the plastic substratum, depending upon the species, and after a given time were observed to form clusters or "chains of beads" as previously described by Spiegel and Spiegel (1975). These detached from the plastic substratum in about 1$\frac{1}{2}$–2 h for *A. punctulata* and *L. variegatus* at 20–22°C; 4–5 h for *A. punctulata* at 16.5°C, and 6–7 h for *S. purpuratus* at 16.5°C. Aggregates of cells were transferred by Pasteur pipette to conical centrifuge tubes and recovered by gentle centrifugation with a hand-driven centrifuge. The aggregates were washed three times in ASW at 0°C by gravity sedimentation and resuspended to the desired concentrations of 0.1–2.5 mg of protein per ml in ASW containing gentamicin and 1 mM leucine. The average size of the isolated collecting aggregates was equal to that of a normal blastula or somewhat larger. Yields of collecting aggregates from single cells of *A. punctulata* and *L. variegatus* averaged 25–60%; those from *S. purpuratus* averaged 25% in 1982–83 but dropped to 5–10% in 1983–84. These yields represent lower estimates of the percent reaggregation of dissociated cells, since many smaller aggregates were discarded during the gravity sedimentations. Collecting aggregates were distributed to siliconized 25 ml Erlenmeyer flasks and stored at 0°C until ready to be used in the collecting aggregate assay (see below). For preparation of aggregates fixed in glutaraldehyde, collecting aggregates at concentrations of 1–2.5 mg of protein per ml were treated in ASW containing 1% glutaraldehyde at 0°C for 5–10 min, gently centrifuged in a hand-driven centrifuge, and washed three times with ASW before resuspension in ASW containing gentamicin and unlabeled leucine.

Labeling of embryos with [³H]leucine; preparation of labeled probe cells

Embryos at the hatched blastula stage (0.25–0.3 ml packed volume) were recovered by centrifugation by hand or allowed to settle by gravity sedimentation at 0°C. These were resuspended in 10 ml ASW containing Tris and gentamicin and transferred to a 100 × 20 mm plastic Petri dish. Tritiated leucine (carrier-free; 50 μCi) was added, and the embryos were incubated in stationary culture for 2–4 h at the same temperature as the original 1% culture stirred by the paddle wheel. At the end of the labeling period, the embryos were centrifuged by hand and washed several times with 10 ml ASW at 0°C. Aliquots of the recovered supernatant were counted in a triton and toluene-based scintillation fluid. Aliquots of a suspension of the washed embryos were assessed for tritium content by counting and for protein content by the method of Lowry *et al.* (1951). In addition, the amount of [³H]leucine incorporated into protein was assessed in initial experiments by treatment of the washed embryos with ice cold 5% TCA. Under these conditions, it was found that hatched blastulae from all three species took up 70–80% of the labeled amino acid from the medium; moreover, they incorporated 70–90% of the total intracellular label into protein. To ensure that collecting aggregates and labeled probe cells were of the same developmental age, blastulae to be used for probe cells were kept at 10°C while other embryos of the same batch were dissociated and allowed to form collecting aggregates. The cold treatment had no effect on development other than to slow it. In some experiments, portions of the same egg batch were fertilized at an interval of several hours for production of collecting aggregates and probe cells. Both methods gave similar results. To prepare labeled probe cells, 0.25–0.30 ml embryos were washed in 10 ml of ice cold CMFSW containing Tris and EDTA and dissociated as described above. When dissociation was complete, the labeled single cells were recovered by centrifugation at 1200 rpm, washed once in 3 ml of CMFSW, and resuspended in 6–12 ml of CMFSW to a concentration of 1–3 mg of protein per ml (about 1.3–3.9×10^7 cells/ml). Aliquots of this suspension were assessed for tritium and protein content as described above. In addition, aliquots of the supernatants from washing and dissociation of embryos were also counted. By these measurements, it was found that only about 4–12% of the total label in whole embryos was lost during the dissociation procedure for all three species. Moreover, the total label in probe cells (4–6×10^6 dpm/mg, or about 0.3–0.5 dpm/cell) closely correlated with that found in whole washed embryos. Sonicated probe cells were prepared by treatment of dissociated cells in CMFSW at 0°C in a Branson sonifier equipped with a microtip and set at the maximal setting. Complete breakage (absence of cells) was assessed by microscopic examination and required about four to five bursts of 5 s each. For preparation of probe cells treated in glutaraldehyde or formaldehyde, 3.25 ml of ice cold ASW containing 1.23% of the fixative was added to 0.75 ml of the suspension of probe cells in CMFSW. The cells were treated at 0°C for 10 min, recovered by centrifugation at 1200 rpm, and washed once or twice in 5 ml of ASW before resuspension to 0.75 ml. (Formaldehyde was prepared fresh from paraformaldehyde by boiling in distilled water; a concentrated solution of ASW was added to this to obtain the desired amount in ASW.)

Collecting aggregate assay for measurement of cell adhesion

Freshly prepared labeled probe cells (10–50 μl) were added to the Erlenmeyer flasks containing various concentrations of homospecific (or heterospecific) collecting aggregates in a total volume of 2 ml. These were gently shaken on a gyratory shaker (Henkert and Humphreys, 1970) for 2–3 hours at 10–25 rpm. The temperature of
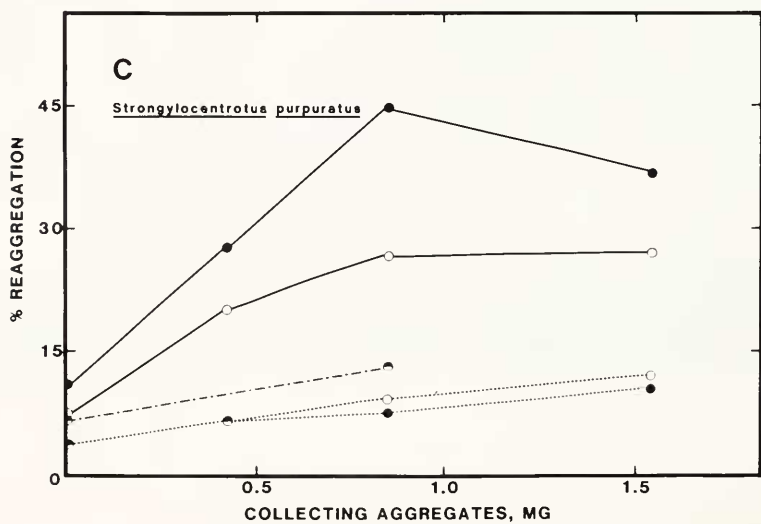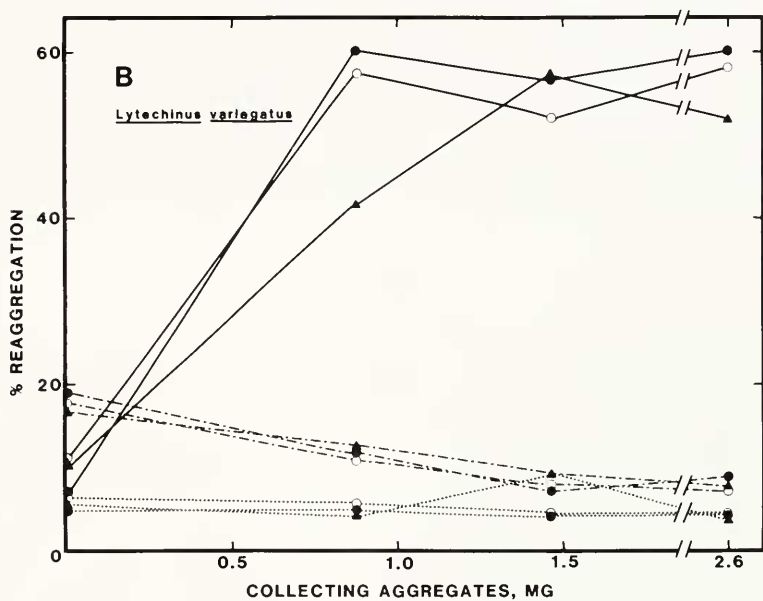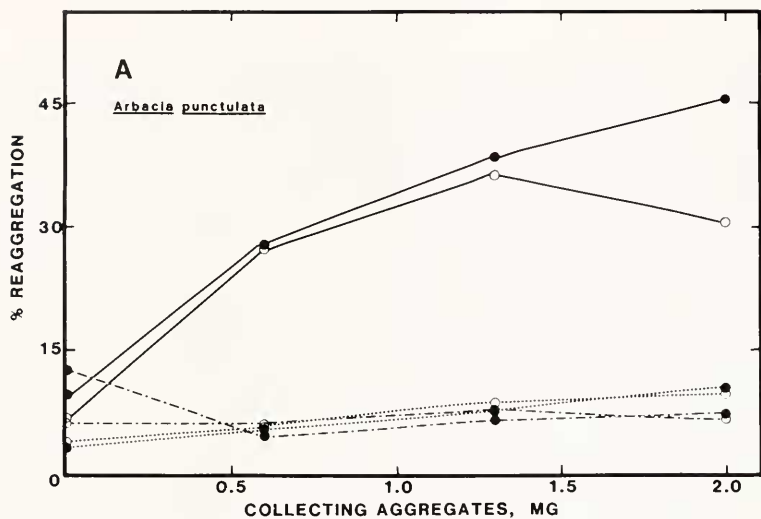
the shaker was controlled in a refrigerated incubator and varied between 16.5°–22°C, depending upon the species used. At the end of the incubation period, the flasks were chilled on ice, and their contents transferred to conical centrifuge tubes. The flasks were washed with 0.5 ml ASW containing 1 mM leucine, and this wash was added to the contents in the centrifuge tubes. Collecting aggregates with adhered probe cells were recovered by hand centrifugation; the aggregates were washed twice with 1 ml of ice cold ASW containing unlabeled leucine. In this manner, three supernatant fractions of 2.5–1.0, and 1.0 ml were obtained; to these were added 10% SDS to a final concentration of 1%. The pelleted aggregates were resuspended in 1 ml of 10% SDS and sonicated. Aliquots of the pellet and supernatant fractions were counted. The percent reaggregation was calculated from the total dpm recovered in the pellet fraction divided by the total dpm recovered (×100%). In nearly all cases, 80–100% of the total dpm added to the flasks as probe cells was recovered. The per cent reaggregation was then plotted as a function of the total amount of collecting aggregates (mg of protein) in the assay flask. Although collecting aggregates with adhered probe cells were routinely washed at 0–5°C, trial experiments in which these were washed at the temperature of the adhesion assay yielded similar results. It was decided to routinely wash at the colder temperatures to help prevent "fusion" of aggregates when these were centrifuged into a small packed volume (a problem especially with aggregates of *S. purpuratus*). In early studies the probe cell concentration was varied in addition to that of the collecting aggregates so that the experimental data represented results from single flasks. In later experiments, the probe cell concentration was held constant so that duplicate or triplicate flasks could be assayed for each concentration of aggregates and results could be expressed as the mean plus or minus the standard error.

RESULTS

Adhesion of labeled probe cells to collecting aggregates

Figure 1 illustrates adhesion of labeled probe cells to their homospecific collecting aggregates for *A. punctulata* (A), *L. variegatus* (B), and *S. purpuratus* (C). A high degree of adhesion is observed for two or three different concentrations of probe cells as a function of the amount of collecting aggregates in the assay, compared to the situations where collecting aggregates were omitted from the assay. In contrast, probe cells which had been sonicated or treated with glutaraldehyde or formaldehyde failed to adhere to the collecting aggregates. Thus, the observed adhesion of probe cells to their homospecific aggregates cannot be due simply to uptake by aggregates of labeled amino acid or proteins from broken or leaky probe cells. In addition, adhesion of probe cells to collecting aggregates appears to require living probe cells. It was of further interest to determine if adhesion in this two-component system also requires living aggregates. Figure 2 illustrates adhesion of *A. punctulata* probe cells to both living and glutaraldehyde-fixed collecting aggregates of *A. punctulata*. Very little adhesion of probe cells to aggregates fixed in glutaraldehyde is observed, in contrast to the living aggregates. Similar data has also been obtained for *L. variegatus* (see Fig. 6) and *S. purpuratus*, although in the latter species, collecting aggregates treated with glutaraldehyde tended to dissociate into single cells, and the recovery of aggregates fixed in glutaraldehyde was sharply reduced (data not shown).

Since adhesion of probe cells to their homospecific aggregates requires both living cells and aggregates, it was important to assess the viability of these in each experiment. As a check on the viability of the probe cells and collecting aggregates, these were routinely returned to Petri dishes containing ASW and gentamicin. The probe cells in all the experiments presented here were observed to reaggregate among themselves
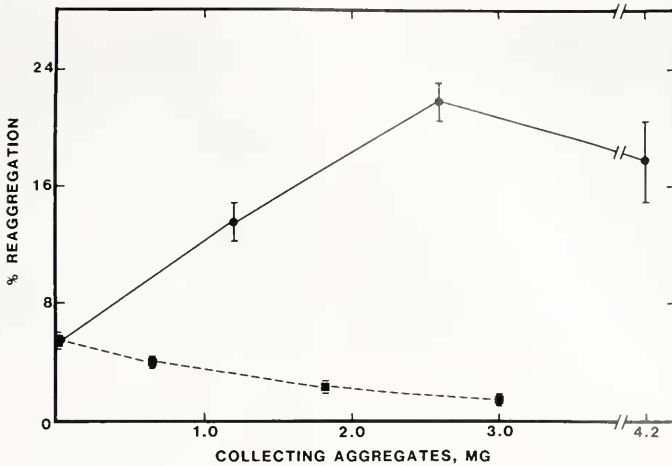
A. Arbacia punctulata — % REAGGREGATION vs COLLECTING AGGREGATES, MG

B. Lytechinus variegatus — % REAGGREGATION vs COLLECTING AGGREGATES, MG

C. Strongylocentrotus purpuratus — % REAGGREGATION vs COLLECTING AGGREGATES, MG

FIGURE 2. Adhesion of labeled probe cells to living and glutaraldehyde-fixed homospecific collecting aggregates. Labeled probe cells from hatched blastulae of *A. punctulata* (7.03 × 10⁶ dpm/mg; 35 μg or 4.5 × 10⁵ cells) were incubated at 16.5°C for 2 h at 10 rpm with the indicated amounts of living (● —— ●) or glutaraldehyde-treated (■ ----- ■) collecting aggregates of *A. punctulata* in a total volume of 2 ml. The data represent results of triplicate assay flasks, plus or minus the standard error of the mean (S.E.M.).

in stationary culture. Moreover, the collecting aggregates were observed to differentiate into embryo-like structures. This process involves transition from a solid mass of adhered cells to blastula-, gastrula-, and finally pluteus-like structures, which are shown for all three species in Figure 3. It can be seen that a very high percentage of these have formed guts, pigment granules, and spicules. Although many of the larger reaggregates contain more than one gut and an abnormal number of arms, a large proportion of the smaller aggregates appear as almost normal plutei. In a few experiments, collecting aggregates without probe cells were shaken for 2–3 h on the gyratory shaker and then returned to stationary culture. Since these were observed also to differentiate into pluteus-like structures, it can be concluded that gentle shaking during the assay does not seriously impair the viability of the collecting aggregates.

FIGURE 1. Adhesion of labeled probe cells to homospecific collecting aggregates. (A) Labeled probe cells (4.01 × 10⁶ dpm/mg) of *A. punctulata* hatched blastulae (● —— ●, 169 μg or 21.8 × 10⁵ cells; ○ —— ○, 68 μg or 8.75 × 10⁵ cells) were incubated at 16.5°C for 2 h at 10 rpm with the indicated amounts of *A. punctulata* collecting aggregates in a total volume of 2 ml. In addition, sonicated probe cells (4.29 × 10⁶ dpm/mg; ● · · · ●, 157 μg; ○ · · · ○, 63 μg) and probe cells treated with glutaraldehyde (3.95 × 10⁶ dpm/mg; ● –·–·– ●, 82 μg; ○ –·–·– ○, 33 μg) were incubated with the indicated amounts of collecting aggregates in a total volume of 2 ml. (B) Labeled probe cells (~4.2 × 10⁶ dpm/mg) of *L. variegatus* hatched blastulae (● —— ●, 144 μg or 17.5 × 10⁵ cells; ○ —— ○, 58 μg or 7.05 × 10⁵ cells; ▲ —— ▲, 29 μg or 3.5 × 10⁵ cells) were incubated at 20–22°C for 2 h at 12 rpm with the indicated amounts of *L. variegatus* collecting aggregates in a total volume of 2 ml. In addition, probe cells treated with formaldehyde (~5.1 × 10⁶ dpm/mg; ● · · · ●, 94 μg; ○ · · · ○, 38 μg; ▲ · · · ▲, 19 μg) and glutaraldehyde (~5.2 × 10⁶ dpm/mg; ● –·–·– ●, 144 μg; ○ –·–·– ○, 58 μg; ▲ –·–·– ▲, 28.9 μg) were incubated with the indicated amounts of collecting aggregates in a total volume of 2 ml. (C) Labeled probe cells (10.3 × 10⁶ dpm/mg) of *S. purpuratus* hatched blastulae (● —— ●, 99 μg or 13.5 × 10⁵ cells; ○ —— ○, 49 μg, or 6.7 × 10⁵ cells) were incubated at 16.5°C for 2 h at 20 rpm with the indicated amounts of *S. purpuratus* collecting aggregates in a total volume of 2 ml. In addition, sonicated probe cells (11.5 × 10⁶ dpm/mg; ● · · · ●, 102 μg; ○ · · · ○, 51 μg) and probe cells treated with glutaraldehyde (8.1 × 10⁶ dpm/mg; ● –·–·– ●, 72 μg; ○ –·–·– ○, 36 μg) were incubated with the indicated amounts of collecting aggregates in a total volume of 2 ml. All points in A, B, and C represent data from single assay flasks.
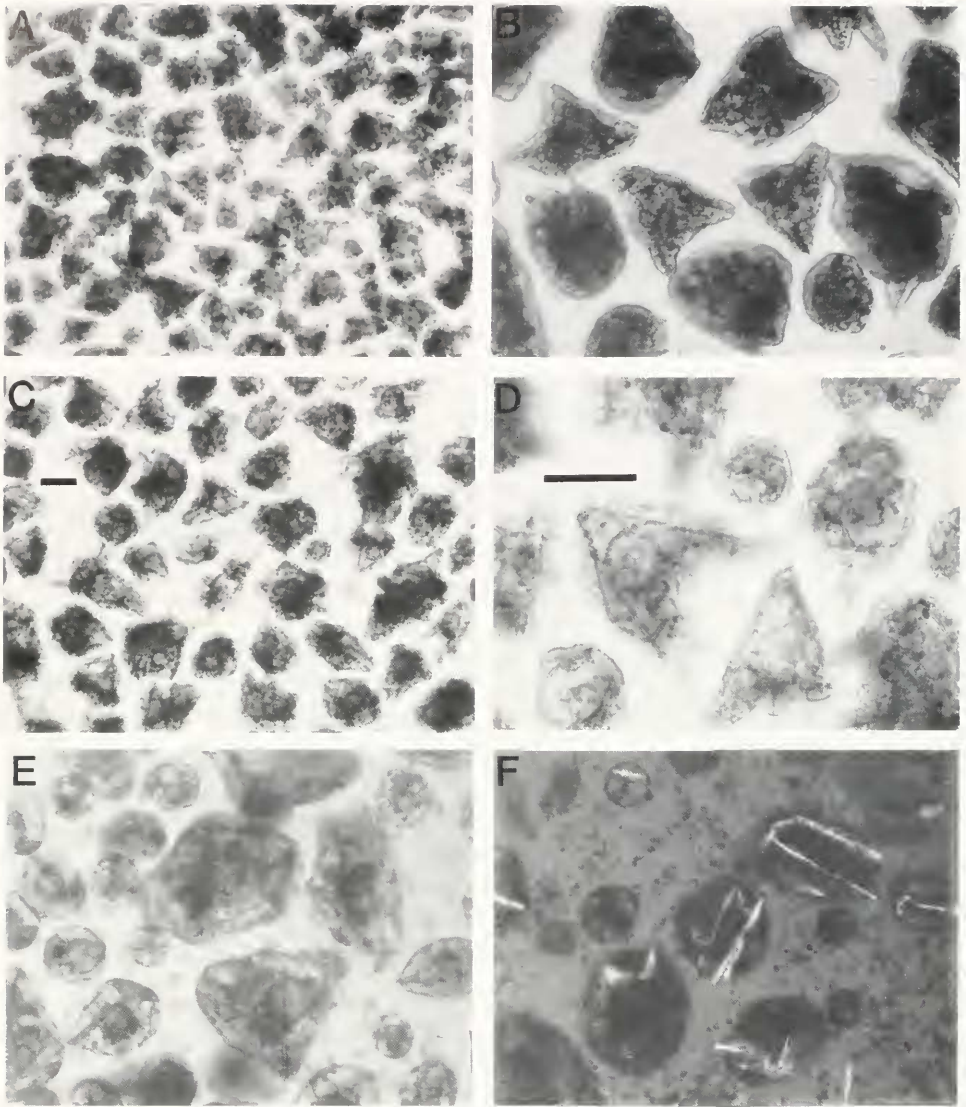
FIGURE 3.   Differentiation of collecting aggregates into pluteus-like structures. A and B: *A. punctulata* embryoids after incubation in ASW in stationary culture for two days at 20–22°C. C and D: *L. variegatus* embryoids after incubation in ASW in stationary culture for two days at 20–22°C. E and F: *S. purpuratus* embryoids after incubation in ASW in stationary culture for four days at 16.5°C. All photographs were taken with the aid of a Wild-Heerbrugg phase contrast microscope with a Polaroid camera attachment. A and C: 40× magnification; B, D, E, and F: 100× magnification. The photograph in F was taken with polarized optics to highlight differentiation of the skeleton (spicules). Scale bars = 100 μm.

Species-specificity of adhesion: reaggregation of probe cells to homospecific versus heterospecific collecting aggregates

Figure 4 illustrates adhesion of labeled probe cells from hatched blastulae of *A. punctulata* to collecting aggregates of *A. punctulata* and *L. variegatus*. A significant
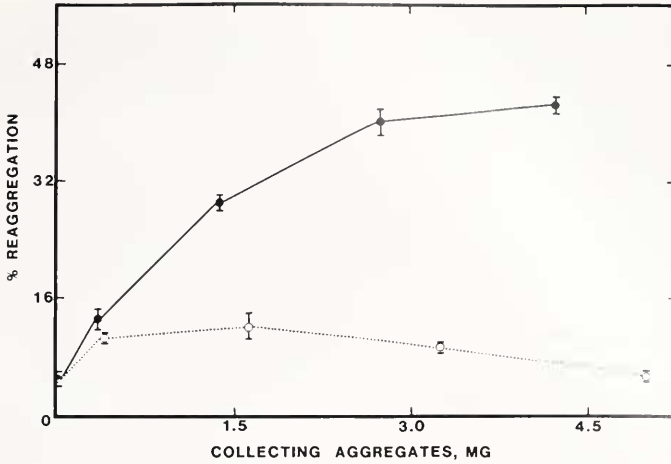
FIGURE 4. Adhesion of labeled probe cells of *A. punctulata* to collecting aggregates of *A. punctulata* and *L. variegatus*. Labeled probe cell from hatched blastulae of *A. punctulata* (4.5×10^6 dpm/mg; 42 μg or 5.4×10^5 cells) were incubated at 18–20°C for $2\frac{1}{2}$ h at 12 rpm with the indicated amounts of collecting aggregates of *A. punctulata* (● —— ●) or *L. variegatus* (○ · · · ○) in a total volume of 2 ml. The data represent results of triplicate assay flasks.

preference for *A. punctulata* collecting aggregates is demonstrated by these probe cells. In a parallel experiment with the same collecting aggregates, probe cells from blastulae of *L. variegatus* demonstrated a preference for collecting aggregates of *L. variegatus* as compared to *A. punctulata* (data not shown).

Figure 5 illustrates adhesion of labeled probe cells from hatched blastulae of *A. punctulata* to collecting aggregates of *A. punctulata* and *S. purpuratus*. A significant
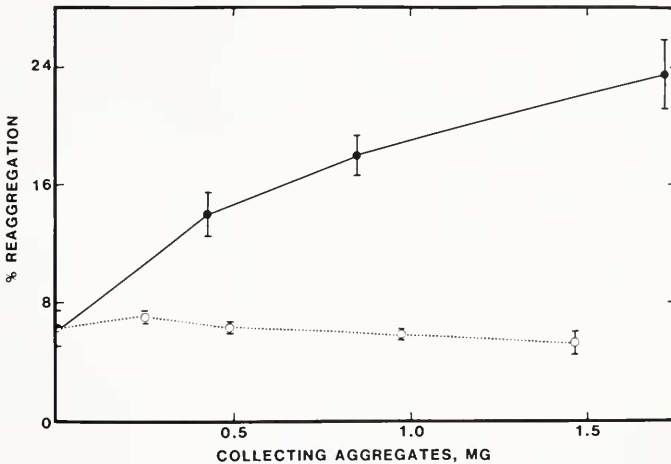
FIGURE 5. Adhesion of labeled probe cells of *A. punctulata* to collecting aggregates of *A. punctulata* and *S. purpuratus*. Labeled probe cells from hatched blastulae of *A. punctulata* (10^7 dpm/mg; 56 μg, or 7.2×10^5 cells) were incubated at 16.5°C for 2 h at 10–12 rpm with the indicated amounts of collecting aggregates of *A. punctulata* (● —— ●) or *S. purpuratus* (○ · · · ○) in a total volume of 2 ml. The data represent results of duplicate assay flasks.

preference for *A. punctulata* collecting aggregates is again demonstrated by probe cells in this experiment. (It should be noted that the percent reaggregation of probe cells to their homospecific aggregates in this experiment reached a maximum of about 28% at 2.5 mg of aggregates. These data are not shown, since there were not enough *S. purpuratus* aggregates available for a comparison.)

Figure 6 illustrates adhesion of labeled probe cells from hatched blastulae of *L. variegatus* to collecting aggregates of *L. variegatus*, *S. purpuratus*, and *A. punctulata*. In addition, adhesion of probe cells to homospecific aggregates fixed in glutaraldehyde is also investigated. It can be seen that a significant preference for *L. variegatus* aggregates compared to *S. purpuratus* or *A. punctulata* aggregates is demonstrated by these probe cells. Although binding of probe cells to *S. purpuratus* aggregates is somewhat higher than to *A. punctulata* aggregates, the former adhesion is no greater than that seen with homospecific aggregates fixed in glutaraladehyde.

Finally, the species-specificity of adhesion of *S. purpuratus* probes to collecting aggregates was investigated in the experiment shown in Figure 7. Here adhesion of labeled probe cells from gastrulae of *S. purpuratus* to collecting aggregates of *S. purpuratus*, *L. variegatus*, and *A. punctulata* is examined. It can be seen that in this species as well, a significant preference for homospecific compared to heterospecific aggregates is demonstrated. Although the experiment in Figure 7 was done with probes from *S. purpuratus* gastrulae, very similar results have been obtained with probes from *S. purpuratus* blastulae and *S. purpuratus versus A. punctulata* collecting aggregates (data not shown).

DISCUSSION

The experiments presented here demonstrate reciprocal species-specific adhesion between *A. punctulata versus L. variegatus*, *A. punctulata versus S. purpuratus*, and *L. variegatus versus S. purpuratus*. These results extend those of Giudice (1962) and
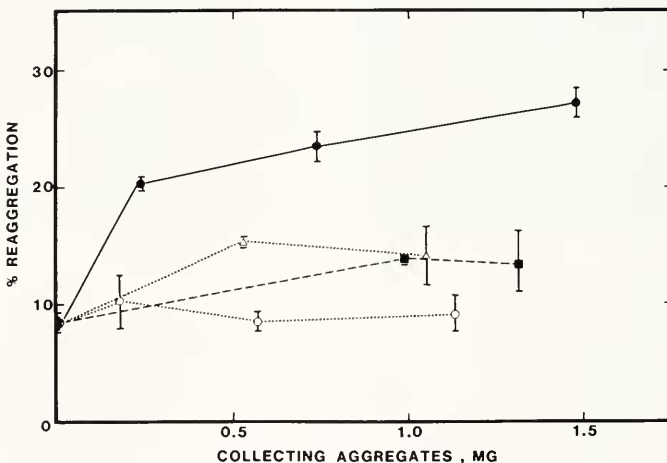

FIGURE 6. Adhesion of labeled probe cells of *L. variegatus* to collecting aggregates of *L. variegatus*, *S. purpuratus*, and *A. punctulata*. Labeled probe cells from hatched blastulae of *L. variegatus* (5.1×10^6 dpm/mg; 16 μg or 1.9×10^5 cells) were incubated at 18°C for 2 h at 20 rpm with the indicated amounts of collecting aggregates of *L. variegatus* (● —— ●), *S. purpuratus* (△ · · · △), or *A. punctulata* (○ · · · ○); and *L. variegatus* collecting aggregates treated with glutaraldehyde (■ ----- ■) in a total volume of 2 ml. The data represent results of duplicate assay flasks.
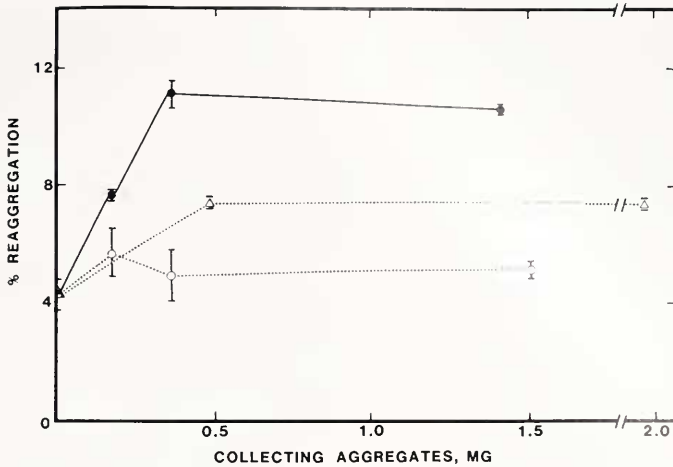
FIGURE 7. Adhesion of labeled probe cells of *S. purpuratus* to collecting aggregates of *S. purpuratus*, *L. variegatus*, and *A. punctulata*. Labeled probe cells from gastrulae of *S. purpuratus* (10^7 dpm/mg; 62 µg) were incubated at 18°C for 2 h at 25 rpm with the indicated amounts of collecting aggregates of *S. purpuratus* (● —— ●), *L. variegatus* (△ · · · △), or *A. punctulata* (○ · · · ○) in a total volume of 2 ml. The data represent results of duplicate assay flasks.

Spiegel and Spiegel (1975) who observed species-specific sorting out between *P. lividus* and *A. lixula,* and between *A. punctulata* and *L. pictus,* respectively. Moreover, these results extend those of McClay and Hausman (1975), McClay *et al.* (1977), and McClay (1982) who demonstrated reciprocal species-specific adhesion between *L. variegatus* and *T. eschulentes* using a quantitative collecting aggregate assay similar to the one used in the present studies. The collecting aggregate assay described here differs in several important aspects from the assay used by McClay and co-workers. First, since the separation of collecting aggregates with adhered probe cells from unadhered probe cells in the present study involves a simple centrifugation procedure, the quantities of probe cells and collecting aggregates in these assays are significantly greater (~ten-fold) than the quantities used in the McClay assay. In addition, the collecting aggregates used in these studies are on the average larger than those used in the studies by McClay. Secondly, the shaker speed in the present studies is 10–20 rpm with a 2″ radius of gyration, while that in the McClay studies is 70 rpm with a 3/8″ radius of gyration (McClay and Baker, 1975). The third important difference in the assay used here compared to that used in the studies by McClay and co-workers is that the present assay measures an end point of adhesion of probe cells to aggregates, not the rate of their adhesion. And finally, McClay and co-workers compared adhesion of different species of probe cells to a single species of collecting aggregates, while in this study adhesion of a single species of probe cell is compared to different species of collecting aggregates.

The present assay offers a few advantages over the assay developed by McClay and co-workers. Since it relies on a simple centrifugation procedure to separate collecting aggregates and probe cells, it eliminates the need for a double filtration procedure and the need to label collecting aggregates with ^{14}C amino acid in order to correct for aggregates which are lost through the Nitex mesh filter and recovered with unadhered probes. According to McClay and Baker (1975) this loss can be appreciable (up to 75%). An obvious disadvantage of the present assay is that it requires much more

biological material. With regard to differences in shaker speed, as discussed by Henkart and Humphreys (1970), the centrifugal acceleration in a gyratory shaker may be increased by increasing either the shaker speed or the radius of gyration. In the present studies we chose to increase the radius of gyration rather than the shaker speed to minimize the increase of liquid shear forces, which have an adverse effect on cell adhesions. In any event, the results presented in Figure 6 for *L. variegatus* probes and aggregates agree favorably with those of McClay and Hausman (1975) and McClay *et al.* (1977) with regard to maximal extent of adhesion of probe cells to aggregates.

Perhaps the most serious objection which might be raised to the specificity measurements reported here is the possible problem of differences in aggregate size between two species. That is, probe cells may adhere to homospecific collecting aggregates simply because these collecting aggregates are smaller and their relative surface area per mg of protein is larger than for heterospecific aggregates. This problem has been discussed in detail in an early paper by Roth and Weston (1967). These authors have observed that larger aggregates collect even fewer probe cells than the *same number* of smaller aggregates, presumably because the larger aggregates remain in orbits further from the center of the flask while the probe cells gather in the vortex at the center. In answer to these objections, it should be pointed out that when the amount of collecting aggregate is varied in the present assay, the degree of adhesion of probe cells is found to reach a constant maximum level. Hence, observed differences in adhesion due to size differences in aggregates of different species should be minimized by varying the amount of collecting aggregates. In these studies it is found that increasing the amount of heterospecific aggregates in the assay never results in the level of adhesion seen with homospecific aggregates. Secondly, the size distribution of aggregates in all three species is very similar, although aggregates of *S. purpuratus* tend to fuse into macroaggregates during the assay compared to the other two species. In addition, the location of aggregates observed in the flasks during the assay is fairly central rather than peripheral; this is probably due to the large radius of gyration and slow shaker speed. Moreover, as shown by Roth and Weston (1967), even when aggregates of different size are used, isotypic adhesions are found to be significantly greater than heterotypic adhesions. Finally, for every experiment presented here, the reciprocal cross was also done at the same time with probes from the other species and the two types of aggregates. In every case, species-specificity was observed. This is certainly evident in Figures 6 and 7, which were from the same experiment. (It should be noted that the probe cell concentrations of *L. variegatus* and *S. purpuratus* were quite different in this experiment).

Species-specific adhesion as measured by the present assay and that of McClay and Hausman (1975) most probably measures the formation of the most stable adhesions, since the washing procedures used by both assays generate enough shear force to remove all but the most stably adhered probe cells. It is not unreasonable to assume that formation of these adhesions may involve several different steps and that some of these may be more dependent on metabolism (and hence temperature) than others. Evidence in favor of this idea has recently been reported by McClay *et al.* (1981). These workers used neural retina cells in an adhesion assay involving binding of labeled probe cells to stable monolayers in microtiter plates and measured the centrifugal force required to dislodge the probe cells when the plates were sealed and inverted. In this study, these workers identified three types of cellular interactions; the first, an initial binding, occurred at 4°C and exhibited recognition specificity. It is noteworthy that recognition specificity is observed as an initial event by this type of assay. In a presentation at the first symposium for Developmental Biology of the Sea Urchin at the Marine Biological Laboratory in August of 1982, D. R. McClay

presented work on sea urchin adhesion using this new monolayer assay which dem-onstrated species-specific adhesion between *L. variegatus* and *A. punctulata* at 0–4°C. Preliminary experiments in this laboratory have also demonstrated species-specific adhesion between these species at 0–4°C using the monolayer assay. These observations support the idea suggested earlier that species-specific recognition is an initial event during reaggregation of sea urchin cells and may be followed by later sorting out of cells in the aggregate according to their tissue specificities.

In recent years Noll and co-workers have extracted dissociated sea urchin cells with n-butanol and identified a factor in these extracts which promotes reaggregation of unextracted cells and is essential for reaggregation of extracted cells (Noll *et al.*, 1979, 1981). However, it is not clear what relationship this factor has to species-specific recognition, since the factor itself is not species-specific and will promote reaggregation of both *P. lividus* and *A. lixula* cells. Recently work by McCarthy and Spiegel (1983) has shown that the enhancement of reaggregation of cells by the butanol extract appears to be relatively non-specific, since dissociation supernatant, human plasma fibronectin, and bovine serum albumin all enhance reaggregation in a con-centration-dependent manner. Moreover, these workers question the presumed ex-tracellular localization of the factor extracted by butanol. On the other hand, Op-penheimer and Meyer (1982) have identified a component in the dissociation su-pernatant of sea urchin cells which appears to be both stage and species-specific.

At present it is unclear whether the cell surface molecules assumed to be involved in species-specific recognition in the sea urchin are localized in the hyalin-containing extracellular matrix, the embryonic plasma membrane, or both. Thus far, components identified in the extracellular matrix such as laminin and fibronectin (Spiegel *et al.*, 1983), and collagen (Spiegel and Spiegel, 1979) have not been isolated from sea urchin embryos and tested for possible species-specific effects in a quantitative reaggregation assay. In addition, plasma membranes from embryos or dissociated cells have not been isolated in highly purified form and tested in a quantitative reaggregation assay. Curent efforts in this laboratory are aimed at such experiments to localize the species-specific reaggregation factors.

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